Role of PKCβ in Breast Cancer

Undergraduate Honors Thesis

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ABSTRACT

The protein kinase C (PKC) family of serine/threonine protein kinases and their tumor-promoting role in cancer research has been studied for nearly three decades. Previous studies showed that the PKC gene plays a major role in signal transduction and regulation of gene expression, and identified PKC activators as tumor-promoting agents. The PKCβ isoenzyme, in particular, has been linked to various types of cancer through its association with blood vessel formation, which plays a principal role in tumor progression. While the PKC gene has been the focus of a multitude of studies, the mechanistic function of many of its isoforms is not yet wholly understood.

This study focuses on gaining insight on the function of the PKCβ isoenzyme through the use of a genetic mouse model. We aim to determine a possible mechanism through which PKCβ promotes tumor growth. Furthermore, we seek to gain a better understanding as to whether the main role of PKCβ in tumor progression is cell-autonomous or whether it interacts with the tumor microenvironment. Ultimately, better mechanistic insight into the function of this tumor-promoting gene can lead to the development of more effective inhibitor treatments for cancer therapies.

ACKNOLEDGEMNTS

First of all I would like to thank Dr. Ostrowski for giving me the opportunity to work on this project. In addition I would like to thank him as well as Julie Wallace, Nandini Sharma, and the other members of the Ostrowski lab for their guidance, mentorship, and support throughout the past three years. I would also like to thank Lianbo Yu from the Biostatistics Lab for conducting the data analysis for this project, and the other members of my thesis committee, Dr. Acton and Dr. Booton for taking time out of their busy schedules to provide me with their support. Finally, I am gratefully indebted to the Pelotonia Fellowship Program for providing funding for this research.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1 - INTRODUCTION

Protein kinase C (PKC) is a family of serine-threonine kinases, consisting of several isoforms. Their main purpose is to help regulate various cell physiological processes and functions, including proliferation, differentiation, apoptosis and migration. In general, they are involved in facilitating the interaction of the cell with its environment, thus maintaining intracellular homeostasis [1]. Misregulation of these kinases leads to cellular proliferation, which in turn can lead to tumor formation. Understanding the precise mechanisms though which these kinases act to impact tumor formation and progression would likely yield the knowledge necessary to counter their misregulation and thus inhibit the respective aberrant behaviors. Ultimately, that would lead to the development of more effective cancer treatments and therapeutic strategies.

The ultimate goal of any cancer-related research is the eventual translation of the knowledge acquired into a clinical setting. Along these lines, if we are able to determine compensatory pathways that are activated in the absence of PKCβ, dual inhibitor treatments may be more successful for the treatment of patients.

1.1 Background on Biological Function

Since their discovery some thirty years ago, in the 1980's, the family of Protein kinase C enzymes has been the focus of a considerable body of biomedical research. Their basic function, including their activation mechanisms and their role in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues, is well understood. Excellent, comprehensive overviews of their biological function are given in a few articles, including Parker and Murray-Rust (2004) [2].

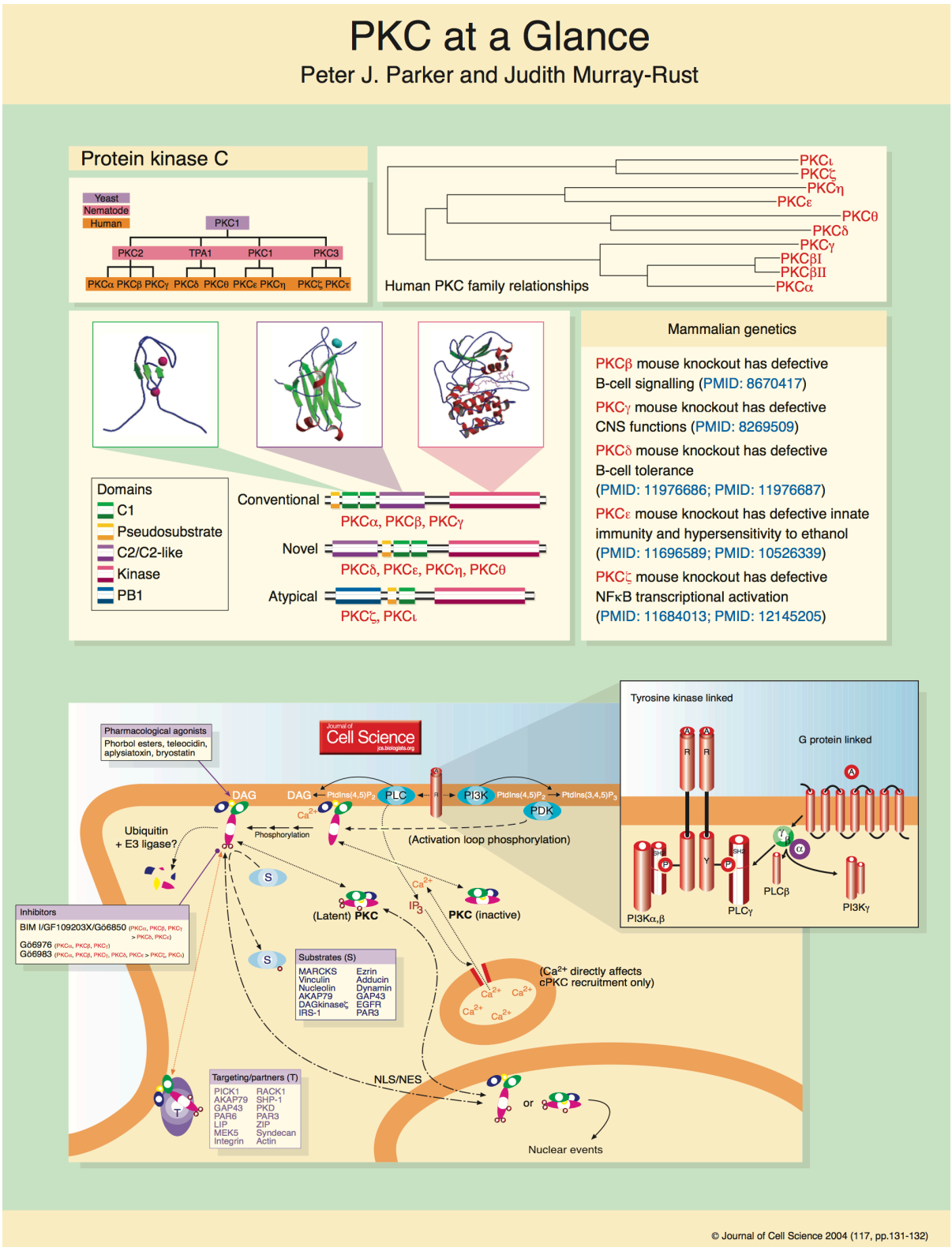

Figure 1: Overview of the Protein Kinase C family of isozymes and their activation pathway. Adapted from "PKC at a glance" by *Peter J. Parker and Judith Murray-Rust.* [2]

There are at least fifteen different currently known isoforms in the PKC family of human enzymes [3]. They are typically organized into three subfamilies: conventional (or classical), novel, and atypical, depending on their activation requirements. The β isoform (PKC β) in particular, which is at the center of our focus in this study, is a member of the conventional subfamily, which requires Ca^{2+}, DAG (diacylglycerol), and a phospholipid for activation. Figure 2, below, provides a sketch of the classical PKC activation pathway in which phospholipase C (PLC) is activated by ligand and in turn generated DAG and IP_3 from membrane phospholipids. DAG then moves PKC to the cell membrane while IP_3 potentiated release of intracellular Ca^{2+}, both of which are utilized to activate PKC [3].

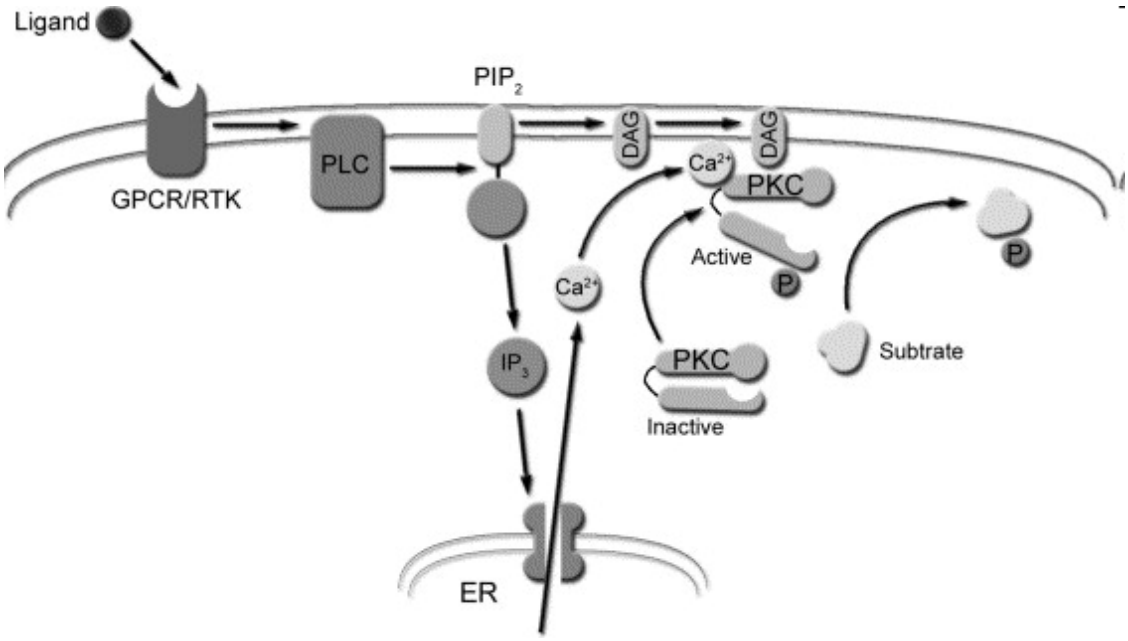

Figure 2: Classical PKC activation pathway. Adapted from "Protein Kinase C(PKC) family in cancer progression" by *Koivunen, J. et al.* [3]

1.2 Review of the role of PKCβ in Cancer-Related Research

The profound role of PKCβ expression in tumor progression has already led to the development and study of a variety of PKCβ-specific inhibitors, which have been tested in multiple cancer studies, including some clinical settings. In the context of human breast cancer, implementing PKCβ targeted therapies can indeed help improve future clinical outcomes [1]. Investigations into the specific roles of PKCβ in breast cancer cells lines has also revealed PKCβ-specific inhibitors such as LY379196 that show reduction in growth of specific breast cancer cell lines including MCF-7, MDA-MB-231, and BT474 [4]. This decrease in viability of cancer cells due to PKCβ specific inhibitors points to a promising future for the field of breast cancer therapeutics.

Over expression of PKCβ was also found in the MDA-MB-436 triple negative basal cell line, which is known as one of the most aggressive and hard to treat subtypes of human breast cancer [5]. Additionally, Vascular Endothelia Growth Factor (VEGF), an endothelial cell mitogen involved in angiogenesis, appears to increase activation of PKC [6]. Investigation of cell line specific inhibitors of the VEGF-induced PKC activation and proliferation is yet another key for future advancements in the field of breast cancer study and therapeutics.

1.3 Hypothesis and Study Objectives

Our goal in this study has been to further investigate, analyze, and understand the role of PKCβ in tumor initiation and progression, with breast cancer specifically as the target in mind. The complexity and lack of detailed understanding of the specifics involved in cell signaling and downstream implications triggered by this particular isoform is further complicated by the variability in the microenvironment of different types of tumors.

Consequently the best that we can hope to accomplish at this point in time is to use laboratory work to establish an association between certain tumor growth behaviors (or the lack of) and the presence (or absence) of PKCβ. The presence (or absence) of certain specific markers identifiable via immunofluorescent staining that could signify particular tumor growth behaviors could be especially informative in helping us establish the mechanisms via which PKCβ activation might effect tumor progression.

At a macro level, our hypothesis is that deletion of PKCβ, and consequently effective inhibition of the downstream implications from its activation, would result in delayed tumor initiation and reduced tumor progression. At the micro level, we expected to see a significant reduction in microphages, endothelial cells, and/or cell proliferation as a result of PKCβ deletion; thus providing an explanation for the observed difference between the two genotypes in their underlying level of resilience.

Finally, in designing our study, we aimed to understand whether PKCβ function in the tumor cells is cell-autonomous or whether its function is also important in the tumor microenvironment.

CHAPTER 2 - MATERIALS AND METHODS

Given our goal to investigate the role of PKCβ in breast cancer initiation and progression, the first task we needed to accomplish was to identify a suitable animal model. For that purpose we adopted the mouse model of breast cancer caused by expression of the polyoma middle T oncoprotein (PyMT) in the mammary epithelium proposed by *Lin et al* [7]. As depicted in Figure 3, this PyMT mice model indeed exhibits great morphological similarities with human breast cancer, including four distinctly identifiable stages of tumor progression from premalignant to malignant. Those resemble quite closely human breast diseases classified as benign or in situ proliferative lesions to invasive carcinomas.

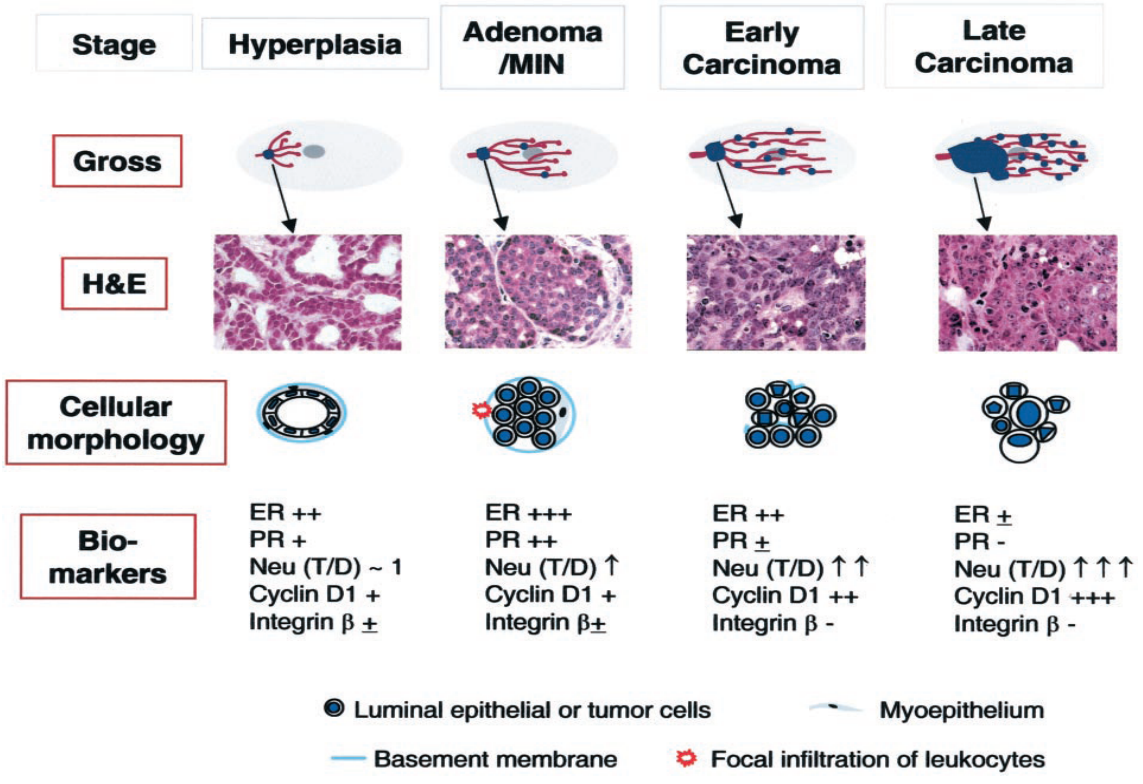

Figure 3: The PyMT model of "breast"/mammary cancer. Adapted from "Progression to Malignancy in the Polyoma Middle T Oncoprotein Mouse Breast Cancer Model Provides a Reliable Model for Human Diseases" by *Lin et al.*

Beyond the morphological similarities, the expression of biomarkers in PyMT-induced tumors is also consistent with those in humans; thus providing a mouse model that is an excellent tool in understanding the biology of breast cancer tumor progression in humans.

2.1 Experimental Unit Selection – The PyMT/PKCβ Genetic Mice Model

Relying on the PyMT mice model, described above, we used genetic breeding to cross PyMT male mice with either their knockout (experimental) or wild type (control) female non-PyMT counterparts as shown in Figure 4, below, to produce both our "control" and "experimental treatment" subjects.

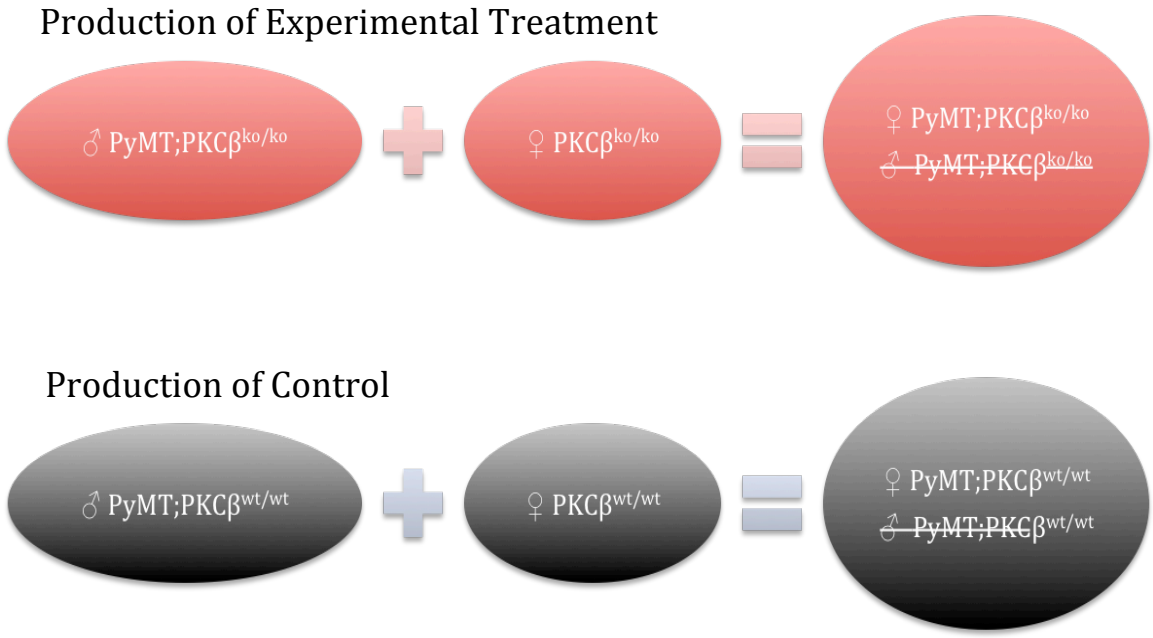

Figure 4: Genetic breading scheme used to produce experimental (treatment) and control units for the study; only the female offspring were used in our experiment.

Limiting the PyMT oncogene to only the male parent mice, in the breeding cages, was simply a matter of convenience to minimize the likelihood of the parent mice developing tumors, as the PyMT oncogene principally effects *mammary* tumor development and progression in females.

2.2 Genotyping

To identify which mice in the breeding scheme were the mice of interest for each study, the mice were labeled and genotyped as pups. At approximately ten days old the mice were numbered and marked by litter and at the same time a small piece of their tail was cut to be used for genotyping.

DNA Isolation - The tail sample was collected in an Eppendorf tube and digested in protienase K and lyse buffer overnight. To extract the genomic DNA from the tails we followed an isopropanol precipitation protocol (see, e.g., appendix A).

PCR Amplification - In order to identify which mice carried the PyMT oncogene and whether they were $PKC^{wt/wt}$ or $PKC^{ko/ko}$ we utilized Polymerase Chain Reaction (PCR) technique to amplify regions of the genomic DNA extracted from the tails. This technique uses specific thermal cycling conditions and primers that are complimentary to each of the sense and anti-sense strand of the DNA region targeted for amplification. In addition to the primers, PCR requires a DNA template (extracted DNA from the tails) containing the target region, Taq polymerase (an enzyme used to cut the genome of the extracted DNA), dNTPs (nucleotides which serve as building blocks for the polymerase to synthesize new DNA strands), and a buffer solution (stable environment for reaction).

Gel Electrophoresis - Products obtained from the PCR amplification were run on a 2% ethidium bromide, agarose gel and a UV light camera was used to visualize and denote the DNA fragments.

As part of our study we carried out two separate and distinct experiments. Nevertheless, the genetic breading scheme described above was used to produce the experimental subject units for both. The study design protocols for those two experiments are provided, below, in the remaining of this chapter.

2.3 Genetic Model Protocol and Study Design

The experiment in this initial phase of our study was designed to help us establish a link between the presence of the PKCβ gene and tumor progression, resulting in higher observable tumor burdens and volumes. In short, we were looking to see raw differences in tumor growth between mice with the PKCβ genotype and mice without the PKCβ genotype, which would then help us establish such a link. For that purpose, we used 32 female mice subjects, 16 each from the control and experimental genotypes bred according to the scheme outlined above (see e.g., Figure 4). Mice entered our study as they were bred, sequentially.

Starting at three months after birth, mice were palpated bi-weekly until palpable tumors were felt. Subsequent to that point, we allowed an additional three weeks for the tumors to progress before we harvested the mammary glands from the mice for observation and measurements. The study design for this experiment allowed us to measure both the length in

time it takes the mice to develop palpable tumors, as well as the rate of progression of the tumors over the three-week period subsequent to the initial detection.

At the time of harvest, tumor burden was measured as a ratio of tumor weight/total body weight of the mouse. Tumor volumes were calculated by means of the product of the measured tumor length (l), width (w), and height (h). Each of the tumors were then either frozen or fixed in formalin for subsequent histological and DNA, RNA and protein analyses.

2.4 Tumor Injection Protocol and Study Design

Capitalizing on our findings from the first experiment, in this second phase of our study we shifted the focus of our attention to determine whether the function of PKCβ, if any, is tumor cell autonomous or cell non-autonomous. For that purpose we injected three million tumor cells into the fat pad of mammary glands 4 and 9 of female mice, consisting of: (i) a group of 13 mice which had the wild type $PKC\beta^{wt/wt}$ genotype (e.g., the control sample), and (ii) a group of 12 mice which had the knockout $PKC\beta^{ko/ko}$ genotype (e.g., the experimental treatment sample). Both the control and the experimental treatment mice were bred in much the same way as the scheme outlined in Figure 4, with the only difference that neither the males nor the females in the breeding cage carried the PyMT gene. The subject mice were introduced into our study in mixed batches, sequentially as they were bred, once they reached the age of six weeks old.

We obtained the tumor cells used for the fat pad injections from another member in our lab. The cells were harvested from B6 mice carrying the PyMT ongogene but not the PKCβ gene. The cells were initially obtained frozen in solution and to acquire the six million cells needed per mouse, we plated the thawed cells and cultured them in a 37°C incubator for approximately a week or two before the scheduled injection. The cells were typically split three

to four times, following standard cell culture passage protocol, at a 1:3 ratio until we accumulated enough cells for a particular batch of mice. At that point, cells were collected and injected into the fat pads of mammary gland 4 and 9.

Tumor progression was monitored through palpation once a week following the injection. Tissue was harvested from entire batch of mice on the first instance we could detect a tumor of at least one centimeter in size, in at least one of the mice in the batch (typically about two-three weeks after the injection). In all ways, we used the exact same harvesting protocol described in previous section and the tumors collected either frozen or fixed in formalin for future analysis. At the time of analysis, sections were cut from the tumor harvested from mammary gland 9, which was fixed in formalin. These sections were then stained: (i) via the Meca32 antibody for angiogenesis, (ii) via the Ki67 antibody for proliferation, and (iii) via the F4/80 antibody for macrophage activity.

2.5 Immunofluorescent Staining Protocol

Our next step was to characterize the tumors collected from both the genetic and injection studies to help determine a possible mechanism through which PKCβ may be promoting tumor growth. This characterization was carried out through immunoflurescent staining for markers of proliferation (Ki67 antibody), macrophages (F4/80 antibody), and angiogenesis/endothelial cells (Meca 32 antibody). To compare differences between the experimental group and control group tumors we selected tissue sections from three pairs of mice.

The pairs of mice for the genetic tumor study, three PyMT;$PKC^{wt/wt}$ and three PyMT;$PKC^{ko/ko}$, were selected and paired together based on date of birth, so that each pair was roughly the same age. The pairs of mice for the injection tumor study, three $PKC^{wt/wt}$ and three

$PKC^{ko/ko}$, were selected and paired together based on date of injection, so that each pair was injected at the same time. The tissue sections used for staining were cut from the mammary tumors fixed in formalin, which had been preserved with paraffin wax and mounted on slides by our histology department.

For staining analysis of the macrophages, proliferation, and angiogenesis markers we followed a standard immunofluorescent staining protocol (see, e.g., appendix B). Once all the sections were stained, we photographed them using a fluorescent microscope. We took five pictures for each slide, for a total of sixty, for each marker of interest. To quantify for proliferation we utilized a counting tool in Adobe Photoshop to hand count the total number of proliferating cells (positive staining probed by Ki67 antibody) in each picture as well as the total number of nuclei which were stained by DAPI (as noted in the protocol). The ratio of the total number of macrophages to the total number of nuclei was then converted to a percentage. The same analysis was used to quantify the percent of macrophages for the slides probed with the F4/80 antibody. By contrast, for the quantification of angiogenesis we utilized the a photo analysis program called Fiji, which measured the percent of positive staining, probed by the Meca 32 antibody, for each picture.

CHAPTER 3 - RESULTS

Our research in this study was organized around two separate experiments. The first of those experiments was designed to help establish a link between the presence of the PKCβ gene and tumor progression, focusing on raw differences in tumor growth between mice with the PKCβ gene and mice without the PKCβ gene. Building on the findings from the first experiment, the second one shifted the focus of attention to the question of whether the function of PKCβ, as it relates to its impact on tumor progression, is cell autonomous or cell non-autonomous.

In summary, the results from the two experiments combined have shown a statistically significant difference in tumor growth between the treatment and control samples. That was true with regard to both the genetic tumor study and the injection tumor study, with the latter further suggesting a probable impact in a cell non-autonomous way. Quantification analysis of the results obtained via immunofluorescent staining also showed significantly decreased proliferation and macrophage activity for the treatment $PKC\beta^{ko/ko}$ group in the Genetic Tumor Study.

3.1 Results from the Genetic Model Tumor Study

Tumor burden was defined as the ratio of total tumor weight (across all ten mammary glands) over the total body weight of the mouse. The raw data measurements obtained from the experiment, in this part of the study, on tumor burden are displayed graphically in Figure 5. A summary of the statistical analysis of the same data comparing the treatment against the control samples, using the Mann-Whitney Test, is also provided in Figure 6. With a p-value of 0.0418

there is indeed enough evidence to establish statistical significance (at the α=0.05 significance level); thus pointing to a link between PKCβ and mammary tumor growth. Nonetheless, it should be noted that the observed difference is only marginally significant due perhaps to our small sample sizes and excessive noise resulting from our data collection approach (see also Chapter 4 for more discussion).

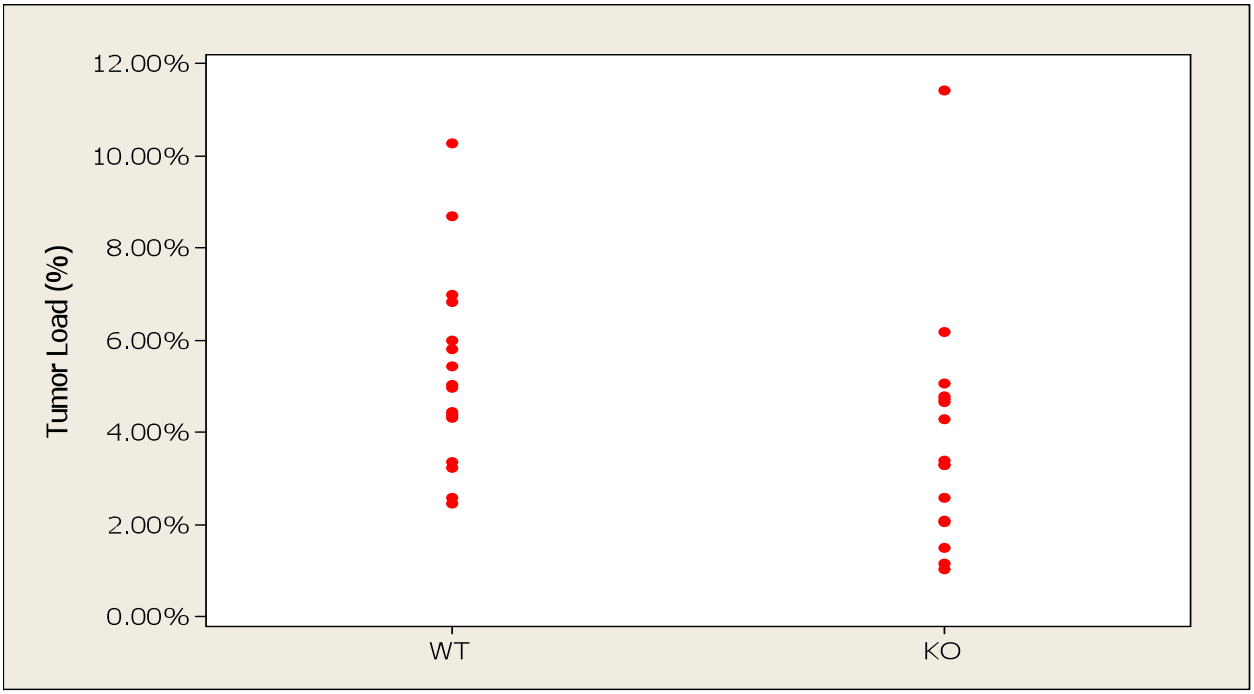

Figure 5: Data obtained from the Genetic Model Tumor Study show a clear shift in the distribution of measurements with significantly lower tumor burden values for most of the mice with the PKCβ$^{ko/ko}$ genotype, except from the case a single potential outlier.

```
Mann-Whitney Test and CI for Tumor Burden

     N   Median
WT  16  0.05010
KO  16  0.03346

Point estimate for ETA1-ETA2 is 0.01573
95.2 Percent CI for ETA1-ETA2 is (0.00063,0.03185)
W = 318.5
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.0418
The test is significant at 0.0418 (adjusted for ties)
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Figure 6: Mann-Whitney Test for the Tumor Burden Measurements in Genetic Model Tumor Study shows a statistically significant difference between PKCβ$^{wt/wt}$ and PKCβ$^{ko/ko}$ mice.

3.2 Results from the Tumor Injection Study

Given the design protocol for this experiment, tumor volume may be the best quantitative measurement to represent tumor progression. The raw data obtained on tumor volume, in this part of the study, are displayed graphically in Figure 6, and a summary of the statistical analysis comparing the treatment against the control, using the Mann-Whitney Test, is provided in Table 2. With a p-value of 0.0471 there is enough evidence to establish statistical significance (at significance level of α=0.05); thus further confirming a link between PKCβ and mammary tumor growth even when the PKCβ gene is only present in the tumor microenvironment, i.e., the impact in this case is the result of cell non-autonomous function (see also Chapter 4 for more discussion).

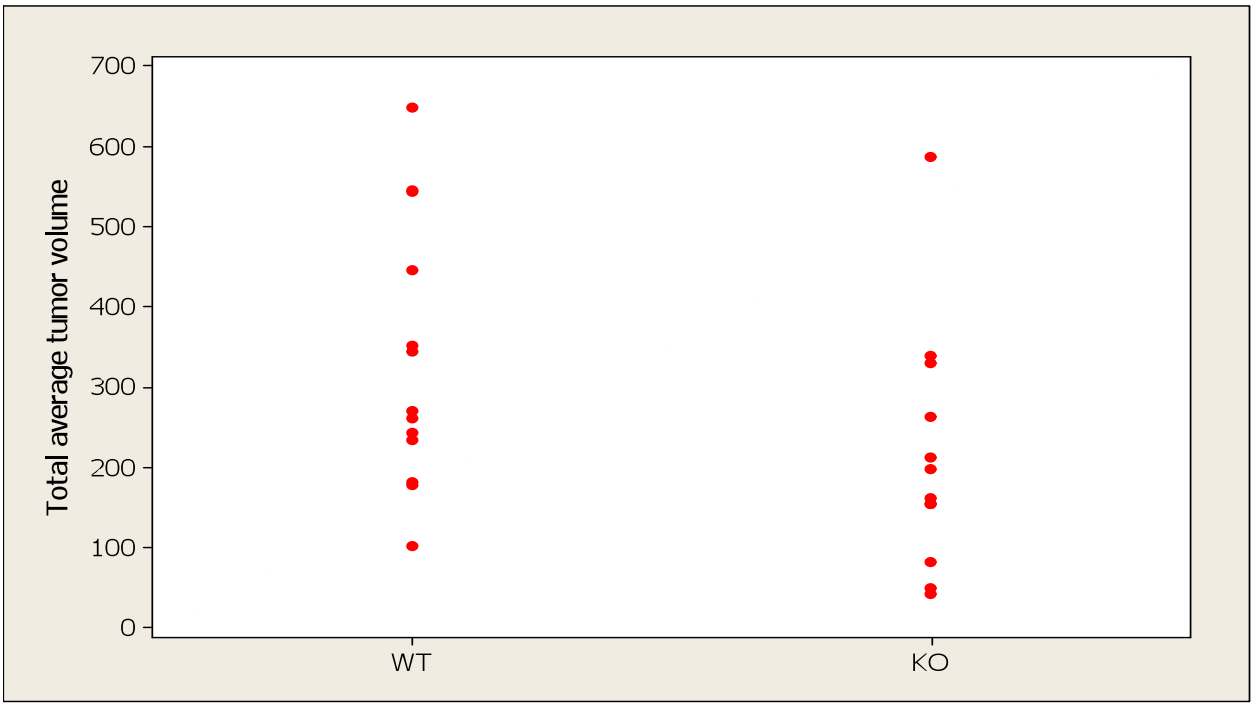

Figure 7: Data obtained from the Injection Study shows a shift in the distribution of tumor volume, with significantly lower volumes for the PKCβ$^{ko/ko}$ genotype.

Mann-Whitney Test and CI for Average Tumor Volume

```
 N  Median
WT  13   270.6
KO  12   180.1
Point estimate for ETA1-ETA2 is 108.4
95.3 Percent CI for ETA1-ETA2 is (4.3,248.5)
W = 206.0
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.0471
```

Figure 8: Mann-Whitney Test for the Average Tumor Volume Measurements in Injection Tumor Study shows a statistically significant difference between PKCβ$^{wt/wt}$ and PKCβ$^{ko/ko}$ mice.

3.3 Results from the Immunofluorescent Staining Quantification Analysis

Quantification analysis of the results obtained via immunofluorescent staining showed significantly decreased proliferation and macrophage activity for the treatment PKCβ$^{ko/ko}$ group in the Genetic Tumor Study, but no difference in angiogenesis. No differences, whatsoever, were observed in the Injection Tumor Study.

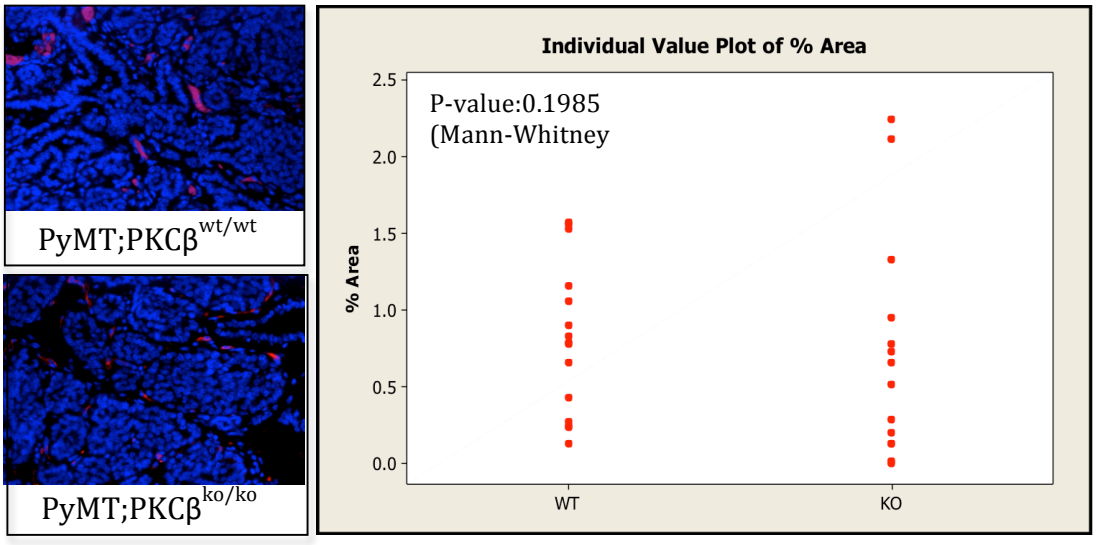

Figure 9: Quantification for angiogenesis via immunofluorescent staining using the Meca32 antibody has shown no significant difference in the Genetic Tumor Study

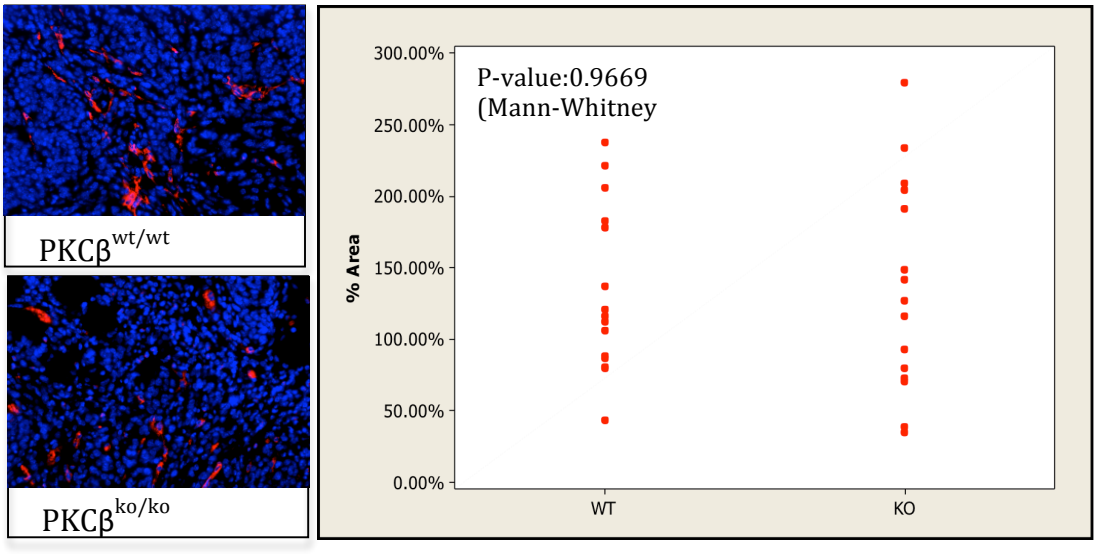

Figure 10: Quantification for angiogenesis via immunofluorescent staining using the Meca32 antibody has shown no significant difference in the Injection Tumor Study

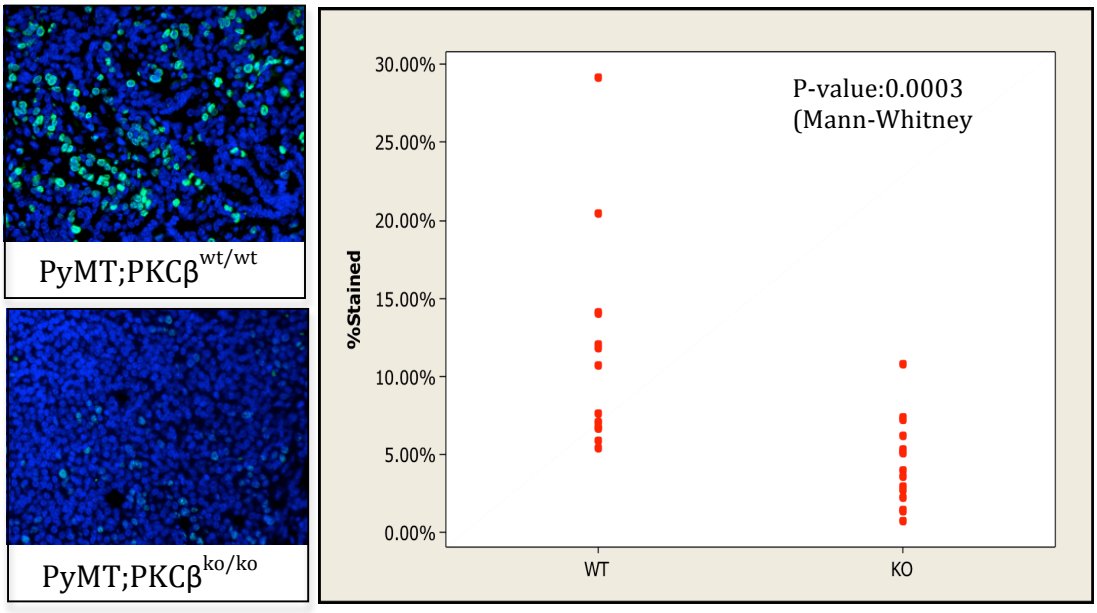

Figure 11: Quantification for proliferation via immunofluorescent staining using the Ki67 antibody has shown a strongly significant difference between the treatment and control mice, in the Genetic Tumor Study, with the treatment group showing significantly lower activity.

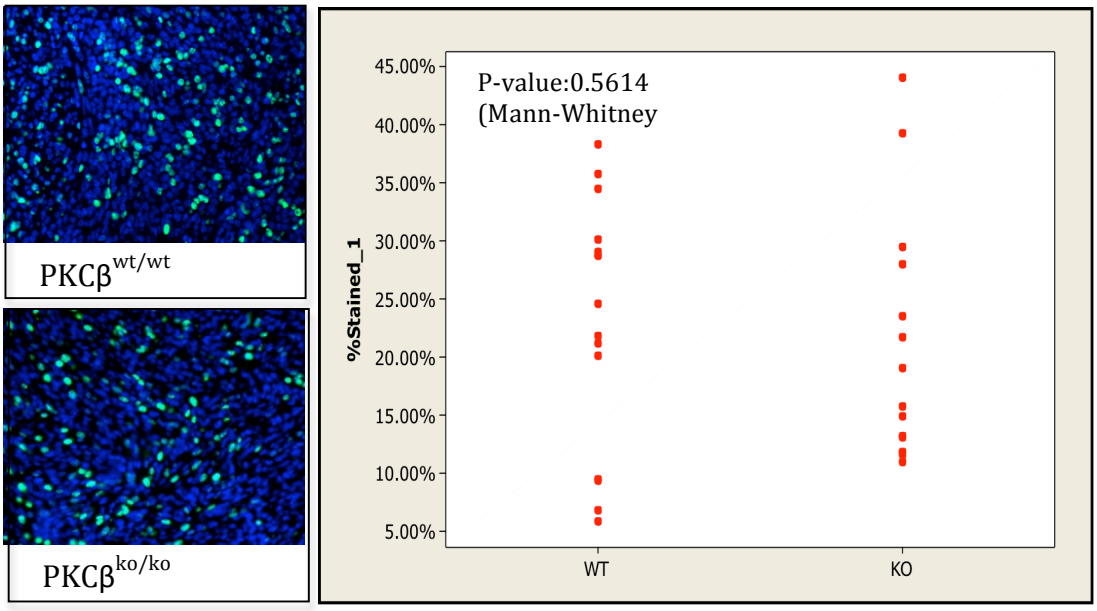

Figure 12: Quantification for proliferation via immunofluorescent staining using the Ki67 antibody has shown no significant difference in the Injection Tumor Study

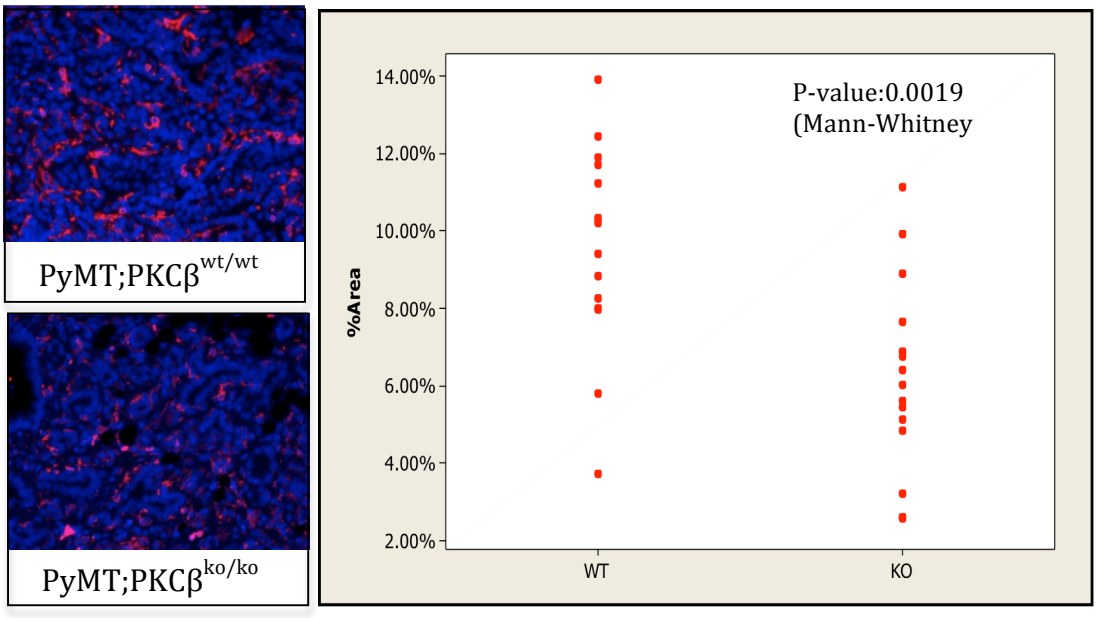

Figure 13: Quantification for macrophage activity via immunofluorescent staining using the F4/80 antibody has shown a significant difference between the treatment and control mice, in the Genetic Tumor Study, with the treatment group showing significantly lower activity.

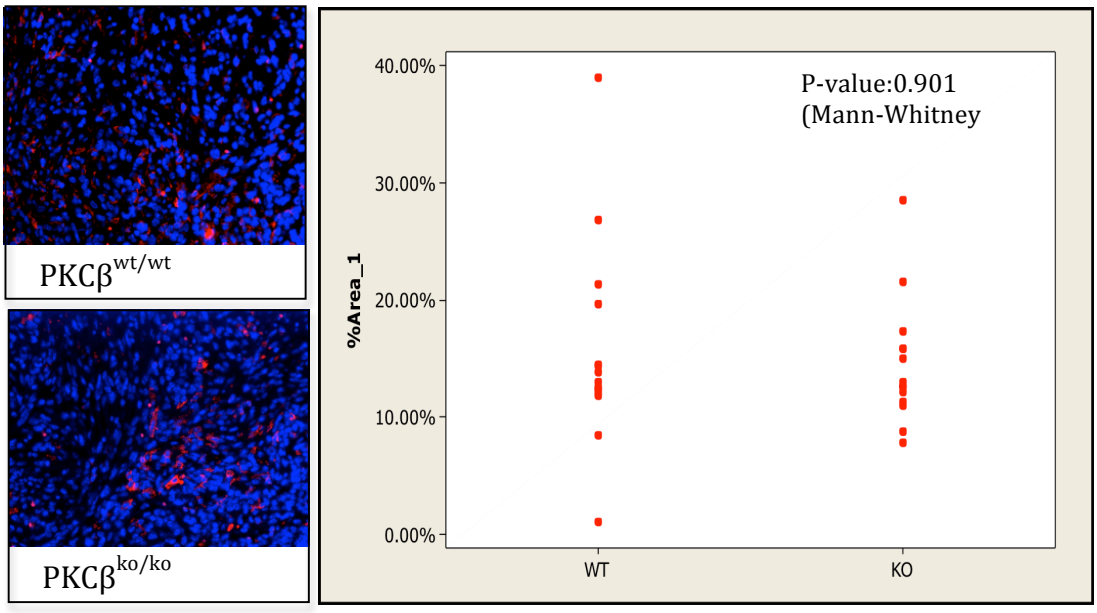

Figure 14: Quantification for macrophage activity via immunofluorescent staining using the F4/80 antibody has shown no significant difference between the treatment and control, in the Injection Tumor Study.

CHAPTER 4 - DISCUSSION

Several recent studies identified a plausible link between PKCβ and the growth of tumor cells for a variety of different cancer types. In much the same way with other PKC isozymes, it is believed that misregulation of these kinases leads to cellular proliferation that can then further lead to tumor formation. Another cell non-autonomous related mechanism that has been a focus of study, with regard to the role of PKCβ, is concerned with endothelial cell proliferation, which is in return critical to tumor angiogenesis.

4.1 Conclusions

With breast cancer therapeutics in mind, as the ultimate objective, in this study we used a mouse mammary tumor model to further explore the potential role of PKCβ in breast cancer progression.

Despite the small scale of our experiments, and in some instances less than perfect data collection protocols, we were able to derive statistically significant (albeit only marginally) results that could be interpreted as a positive link between PKCβ and mammary tumor progression. Nonetheless, these results provide a valuable pilot study for future research. Specifically, we were able to observe significantly lower tumor growth in the experimental treatment subjects, with the PKCβ $^{ko/ko}$ genotypes, as compared to the respective control sample, with the with PKCβ $^{wt/wt}$ genotypes. Even more significant, we were able to replicate these results in a setting (Tumor Injection Study) that would suggest a plausible cell non-autonomous function.

As it was noted in Chapter 3, the fact that some of our results were only marginally significant could be the consequence of a single outlier observation, in each of our two experiments, influencing the strength of the results. Indeed, even a casual inspection of the data in Figures 6 and 7 shows the presence of such suspect outlier cases. The somewhat crude nature of the way we determined the timing to harvest tumors for measurements gives further credence to the suspicion of outliers and increased noise in our data that may have masked the strength of the difference between the experimental treatment and control sample measurements. A repeat study with larger sample sizes and more rigor in the data collection approach would likely yield even more significant results.

Yet, another interesting finding from our study is the fact that we were able to detect significantly decreased cell proliferation and microphage activity in connection with PKCβ $^{ko/ko}$ genotypes in our first experiment (Genetic Model Tumor Study), which provide at least some indication of the underlying function involved. The fact that we were not able to replicate these findings in the second experiment, however, generates another interesting question. The difference in the outcome could be simply the result of the fact that only cell non-autonomous functions could be observed in our second experiment, which could actually be yet another interesting interpretation; nonetheless we must exercise caution in that another interpretation may be simply the fact the results from the staining analysis in the second experiment were impacted by the relatively small sample size and noise in the data collection process.

4.2 Future Directions

While the findings of our injection study conclusively showed a significant difference in tumor progression, indicated by higher burden among the PKCβ $^{wt/wt}$ mice, tumor characterization via

immunofluorescent staining failed to suggest a cause for this difference. Due to the nature of the orthotopic injection study, the results we obtained (based on burden alone) imply that PKCβ in the microenvironment impacts tumor growth thus suggesting a cell non-autonomous function. Future tumor characterization, i.e. staining for other cellular markers, would be necessary to conclusively say whether this tumor promoting gene functions in a cell non-autonomous function for certain.

In order to further investigate the potential cell non-autonomous function of PKCβ in the microenvironment, another prospective study would be to utilize various conditional knockout mice models for different stromal compartments. A repeat of the genetic tumor study conducted in this project could be employed to test the effect of the conditional knockout genotypes on tumor progression, utilizing the various conditional knockout mice as the treatment group.

More recently, some studies have shown a definitive relationship between PKCβ and obesity. The latter, which is a condition of excess adipose tissue, affects over 25% of the US population and has been shown to increase the risk of developing other diseases including diabetes and breast cancer [8]. In the past 10 years, studies have revealed active secretion of cytokines from adipose tissues, which play a key role in processes involved in tumorigenesis. Particularly leptin, one of these cytokines, is thought to accelerate tumor growth. Consequently, this suggests the notion that increased body fat resulting from increased production of these adipokine factors may impact breast tumor growth [8]. Other studies have further illustrated an association between mice lacking PKCβ and reduction in adipose tissue, suggesting PKCβ deficiency may play a key role in preventing genetic obesity [9]. The effect of PKCβ on metabolism is shown by overall reduction in adipose tissue of the PKCβ deficient mice in

addition to resistance to high fat diet induced obesity illustrated by these PKCβ deficient mice as well [10].

To study the relationship between obesity and cancer as related to PKCβ, future studies could use the same genetic model that was employed to study the effect of PKCβ deletion. For example, the same model may be used with a high fat diet induced on half of the subjects to observe differences among tumor growth and progression. Since PKCβ deficiency is suggested to reduce the amount of adipose tissue as well as and show resistance to high fat diet induced obesity, we would expect to see a difference in the rate of tumor progression in this new model. The underlying hypothesis would suggest that the PKCβ $^{wt/wt}$ mice would grow tumors at a faster rate than the mice with the PKCβ deletion. Ultimately that could determine the combined effect of PKCβ on metabolism and tumor progression.

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Appendix A

DNA isolation from mouse tails (isopropanol precipitation)

1. Add proteinase K to lysis buffer, use 400µl lysis buffer per tail(1.5ml Eppendorf tube)
2. Incubate overnight at 55°C
3. Spin samples for 3 min in a microfuge; remove supernatant to new tube
4. Add 500µl isopropanol to supe, mix, let sit for 5 min; meanwhile, prepare Eppendorf tubes with 1 ml of 70% EtOH for next step
5. Remove precipitated DNA with a yellow pipette tip and put in 1ml 70% EtOH
6. Spin for 3min; remove EtOH; dry briefly on benchtop
7. Add 100 µl TAE (best: pre-warm to 55°C)
8. Vortex vigorously to dislodge pellet from the bottom of the tube
9. Put tubes in an Eppendorf shaker for 15+min.
10. Spin for 1 min to pellet extra DNA; use 2 µl supe per 50 µl for subsequent PCR

<u>1x lysis buffer</u>
0.1 M Tris pH 8.0
0.2 M NaCl
5 mM EDTA
0.4% SDS

Just before use, add proteinase K to a final of 0.2 mg/ml (I make a 50x stock by diluting a 100 mg bottle to 10 mg/ml in 50% glycerol; this lasts for months at -20°C).

Proteinase K: stock is 10mg/ml in -20°C. (Proteinase K 100mg; Sigma; P6556)

Preparation: 65°C water bath
warm amount of lysis buffer needed in 65°C water bath to equilibrate thaw
Proteinase K at room temperature

Appendix B

Immunofluorescence of Fixed Paraffin-embedded Tissue Sections

Supplies and Reagents

- Xylene
- Ethanol series
  - 100%
  - 95%
  - 80%
  - 70%
  - 50%
- Heat-mediated Antigen Retrieval Buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0)
  - make and store as a100m M solution, and add Tween 20 prior to use
- Permeabilization Buffer (0.2% Triton X-100 in PBS)
  - make prior to use
- 20X PBS
  - 43.6g Na2HPO4 (sodium phosphate dibasic anhydrous)
  - 12.8g NaH2PO4 (sodium phosphate monobasic anhydrous)
  - 360.0gNaCl(sodiumchloride)
  - bring up to 2L with H2O
  - pH to 7.2
- 1X PBS
  - dilute 20X PBS with H2O
- Blocking Buffer in PBS (use following recipe or alternatively use 10% BSA in PBS)
  - 10% normal serum (host species of secondary antibody)
    - § Horse Serum (Jackson ImmunoResearch 008-000-121)
    - § Goat Serum (Jackson ImmunoResearch 005-000-121)
  - 1%BSA
- Primary Antibody
  - antibody against the protein of interest (γ-H2A.X,activecaspase-3,CD3, etc.) and raised in an animal other than mouse
  - diluted at an appropriate concentration in blocking buffer
- Secondary Antibody
  - antibody against the host species of the primary antibody
  - conjugated to a fluorescent dye (Fluorescein, TexasRed, etc.)
  - diluted at an appropriate concentration in 1X PBS
- DAPI (Sigma D9564-10MG)
  - dilute in PBS
  - store at 1mg/mL
  - use at 0.5µg/mL
- Superfrost/Plus Microscope Slides (Fisher 12-550-15)
- Slide Rack
  - will hold up to 24 slides
- Wash Container

    - slide holder will fit into the container
- Cover Slips 22x30-1.5 (Fisher 12-544-D)
- ImmEdge Pen (Vector Laboratories H-4000)
- Mounting Medium (Sigma G0918-20ML)
- Humidified Chamber
    - can be made with any sealing plastic container and damp paper towels
- Clear Nail Polish (with applicator)

***The longer tissue is fixed in Bouins, the more autofluorescence will result**
***Fixed tissue should have previously been embedded in paraffin and sectioned onto slides**
***All slide washes take place using the slide rack and wash container in 1X PBS**
***A negative control is needed for each run to test the secondary antibodies reaction to the tissue, or background (use protocol as normal, but for one section, replace primary antibody with blocking buffer)**
***Do not allow tissue sections to dry**

I. Deparaffinize
1. Wash slides three times, 5 minutes each, in xylene using glass containers.
2. Wash two times, 5minutes each, in 100% ethanol.
3. Wash two times, 5 minutes each, in 95% ethanol.
4. Wash 5 minutes in 80% ethanol.
5. Wash 5 minutes in 70% ethanol.
6. Wash 5 minutes in 50% ethanol.
7. Wash two times, 5 minutes each, in H2O.

II. Antigen Retrieval
1. Place slide rack into a wash container containing just enough retrieval buffer to cover the slides and cover with lid.
2. Heat in microwave on full power, until boiling starts.
3. Reduce power to 10% or 20% for 9 minutes. **Note: Each time the microwave powers up, boiling should begin and then cease when it powers down.**
4. Remove from microwave and allow to cool at room temperature for 20 minutes.
5. Wash three times, 5 minutes each, in H2O.

III. Permeabilize
1. One at a time, drain slide, but do not let sections dry out.
2. Using the ImmEdge pen, draw a hydrophobic barrier around each tissue section. **Note: to conserve reagents, draw barrier close to, but not on, sections.**
3. Place slide back into H2O, until all slides are done.
4. Wash slides for 5 minutes in PBS.
5. Place slide rack into a wash container with permeabilization buffer for 45 minutes at room temperature.
6. Wash slides three times, 5 minutes each, in PBS.

IV. Block
1. Remove slides from rack, tap off excess PBS onto paper towel, and lay flat in humidified chamber.
2. Add enough blocking buffer to cover each tissue section, using a pipet.

Note: amount of buffers/antibody/DAPI needed will depend on the section size, but is typically less than 100μL per section.

3. Close off humidified chamber and incubate for 1 hour at room temperature.
4. Wash slides three times, 5 minutes each, in PBS.

V. Primary Antibody

1. Tap off excess PBS onto paper towel and lay flat in humidified chamber.
2. Add primary antibody to each section.
3. Close the humidified chamber and incubate overnight at 4°C.

VI. Secondary Antibody

1. Wash slides three times, 5 minutes each, in PBS.
2. Tap off excess PBS onto paper towel and lay flat in humidified chamber.
3. Add secondary antibody to each section.
4. Close humidified chamber, protect it from light, and incubate for 1 hour at room temperature.
5. Wash slides three times, 5 minutes each, in PBS.

VII. DAPI

1. Tap off excess PBS onto paper towel and lay flat on paper towel.
2. Add DAPI to each section.
3. Protect from light and incubate for 10 minutes at room temperature.
4. Wash slides two times, 5 minutes each, in PBS.

VIII. Mount and Cover Slip

1. One slide at a time, remove from PBS and tap off excess onto paper towel.
2. Lay flat on a paper towel and remove any remaining PBS outside of barriers with a folded Kim wipe.
3. Add 3 drops of mounting medium to the slide.
4. While trying to exclude bubbles, lower a cover slip to cover all sections, using a razor blade to position it.
5. Allow to dry for 1 hour at room temperature in the dark.
6. Seal edges of cover slip with nail polish, and allow 15 minutes to dry in the dark.
7. Store slides at 4°C in the dark.